

Cell3™ Direct

Fetal Sex Determination

Analysis Guidelines v1.2



Updates from Version 1.1

1. Paragraph title **Calling male and female outcomes** has been amended.



Intended Use

The analysis process described in this document is intended to be used as a guideline to interpret quantitative/Real-Time PCR (qPCR) data generated by the Cell3™Direct: Fetal Sex Determination kit. These guidelines are not intended to substitute standard operating procedures and we recommend that validation of the kit be conducted on a set number of samples with known outcomes by the end user, in order to assess the best analysis process for data interpretation.

Premise

The following guidelines describe how to analyse qPCR data obtained by following the setup process outlined in the Cell3™Direct: Fetal Sex Determination, Protocol Guide; and in conjunction with the Cell3 Direct - Fetal Sex analysis template excel worksheet (available for download at www.nonacus.com). Therefore it is assumed that 6 replicates of each tested sample were loaded onto the qPCR plate (3 replicates for the ST assay and 3 for the DC assay).

Setting Analysis Parameters on qPCR Instrument and Data Export

Depending on the qPCR instrument used, the associated software interface can differ considerably. However, each software should allow the user to set the analysis settings of their preference. The following settings should be inputted for all targets/dyes:

- Baseline set to automatic*
- Ct threshold for HEX/VIC labelled targets: 0.02 for instruments showing the ΔRn on the Y axis of the amplification plot**; 200 for instruments showing the raw fluorescent units (RFU) on the Y axis
- Ct threshold for FAM labelled targets: 0.04 for instruments showing the ΔRn on the Y axis of the amplification plot**; 400 for instruments showing the raw fluorescent units (RFU) on the Y axis

However, we strongly advise that users find the optimal Ct threshold to use based on their experience with the qPCR instrument in question and the data they obtain. As a



way of roughly determining whether the Ct threshold is in the right range, aim to observe the following Ct values in the positive control:

- TSPY should show a Ct value between 28 and 31
- SRY should show a Ct value between 31 and 34
- DAZ should show a Ct value between 29 and 32
- CCR5 should show a Ct value between 28 and 31

Once the baseline and Ct thresholds are set, export all the data in a .CSV or .xcl format that can be opened with Microsoft Excel.

***NOTE:** occasionally, certain targets may show artefactual/unspecific amplification curves in female/negative samples. In these cases, manually setting the baseline “start cycle” to 5 and the “end cycle” to 25 for these targets may remove these unspecific curves and render the data cleaner.

****NOTE:** in older instruments, the 0.02 and 0.04 Ct thresholds may result too low and should be adjusted to 0.2 and 0.4.

Data Analysis Using Semi-automated Excel Worksheet

Open the Cell3 Direct - Fetal Sex analysis template excel worksheet and select the “Raw Data Analysis” tab. The top of the sheet can be filled in with experiment details. From the results.csv (or .xcl) file containing the data exported from the qPCR instrument, copy over the information contained in the columns of cells titled “Well”, “Sample Name”, “Type (Optional)”, “Target”, “Reporter Dye (Optional)” and “Ct”.

Sort the data so that all the Ct values for the TSPY target are contained between row 14 and row 61 (starting from row 14). Do the same for Ct values for the SRY target so that they are contained between row 62 and 109 (starting from row 62). Repeat the process for Ct values for the DAZ target so that they are contained between row 110 and row 157 (starting at row 110); and for the CCR5 target so that they are contained between row 158 and 205 (starting at row 158).

Sort the data again by sample name for each one of the four targets. This way, the “Analysed results” boxes on the right hand side of the screen will display the mean Ct



value and standard error for each sample, as well as the number of positive and negative replicates identified. This identification is calculated in column I, where Ct values are copied over only if they are lower than 40 and higher than 24. Any Ct values higher than 40 are considered as unspecific amplification; while Ct values lower than 24 are considered artefacts.

NOTE: the 24 and 40 Ct thresholds for discriminating a positive from a negative replicate were obtained empirically and it is ultimately the decision of the user on whether to use them as advised in these guidelines or change them.

Calling Male and Female Outcomes

The analysed data from the “Raw data analysis” tab is automatically copied over to the “Outcomes and graphs” tab in a summarised form. The amount of positive replicates identified for the TSPY target (3 replicates in total), SRY target (3 replicates in total) and DAZ target (3 replicates in total) is represented in percentages in the “Replicates used (%)” box. Based on these values, a calling algorithm will determine whether the sample is:

- Female: 2/9 replicates or less are identified as positive;
- Male: 5/9 replicates or more are identified as positive;
- Inconclusive (Inc): between 3/9 and 4/9 replicates are identified as positive.

This algorithm has been developed empirically and has been used to call outcomes for 50 plasma samples used in our initial validation. However, it is not meant to replace existing classification approaches of individual users and it is ultimately up to them to determine the thresholds to use for data interpretation.

A histogram of the summarised data containing mean Ct values and calculated standard error bars for all samples tested is also included for the user’s convenience.



Internal Control: CCR5

CCR5 is a housekeeping gene commonly used as an internal quality control target in qPCR assays. We have included 1 replicate for CCR5 in the Cell3™ Direct: Fetal Sex Determination kit. As this is a general target, it will amplify both fetal and maternal DNA. Therefore, in most cases it will produce a lower Ct value than the male specific targets. The intended use of this target is to determine the overall quantity of cell free DNA present in the sample and to ensure that the qPCR reaction is working direct from plasma. Samples showing a Ct value higher than 40 for CCR5 should be handled with caution, as this could mean that the qPCR reaction has performed poorly or that there is not enough cell free DNA concentration within the sample. In these cases, cell free DNA extraction from 1 ml of plasma is advised. The Cell3™ Direct: Fetal Sex Determination kit can then be used following the cell free DNA protocol to determine the fetal RhD status.

Artefactual and Unspecific Amplification Curves

Due to the diversity in molecular make up of plasma samples, artefactual or unspecific amplification curves may occasionally be observed. These curves can be easily identified in the amplification plot graph, as they tend to start considerably earlier than genuine amplification curves and proceed in a non-logarithmic and more linear fashion. This type of unspecific amplification usually occurs in female/negative samples. Setting the baseline start and end cycles manually as indicated previously should remove most artefactual amplification curves. However, in the event that one or more of these curves is still present, make a note of the well in which it occurs and the target, and delete the resulting Ct value from the analysis.

Practical Notes on How to Use the Excel Analysis Worksheet

The Cell3 Direct - Fetal Sex analysis template excel file is a semi-automated worksheet intended to make data analysis and interpretation easier and faster for the user. The cells where analysed data appears contain formulas and algorithms which apply to set cell



positions where the raw data is copied over by the user from the exported results.csv (or .xcl) file. Therefore, said data should be inputted only in the appropriate cells as explained in this guide. It is important that cell content is moved by copying, pasting and deleting cells. If cells are cut and pasted or moved by dragging the cells in different positions in the worksheet, this will alter the cell position to which the formulas and algorithms apply to, resulting in incorrect calculations. It is advised that a new copy of the Cell3 Direct - Fetal Sex analysis template excel file is saved prior to starting data analysis, so that the template file remains unaltered and can be used to repeat the analysis when needed.

